

## Making an experiment

In addition to the .rif file format, it is possible to save your file as FCS files, which enables you to view your data (not images) in FlowJo or FCS Express.

To do that, go to "File" in the upper left corner of the Inspire window and choose "save as FCS file". When a check mark appears an FCS file will be saved as well as the .rif file.

Be aware that this function is active until it is unchecked again.

Bring your samples in an eppendorf tube with 30-200 µl sample (preferred concentration 1 mill in 50µl), remember to open the lid on your tube before you load your sample.

1. Select the standard template (file -> "load template" -> Desktop -> General template\_V02.ist) or your own template from a previous date (file -> "load template" -> desktop -> New export folder -> "your name" -> "your template")
2. Before you run any of your samples, load a sample containing 200 µl H<sub>2</sub>O and let it run for a few minutes. Press load, to let all 200 µl run through the ImageStream.
3. In the right side of the screen – click "view all".
4. Select brightfield channels (normally 1 and 9).
5. Chose a channel for side scatter (channel 6 or 12, or turn off the SSC laser, if both channel 6 and 12 are used to detect fluorochromes).
6. Turn on the appropriate lasers for the fluorochromes in your experiment and set laser power to max (NOT for the SSC laser).
7. Select channels by clicking on one of the channels (Ch1-12 listed above the image-area). This will delete all non-relevant plots in the template.
8. Choose magnification: 20X, 40X or 60X and speed.
9. Make a folder to export your data into (Desktop -> New export folder – "your name" – make a folder with today's date).
10. Click the folder in the upper right corner and choose the folder to save your data in.
11. Load your all-stained sample (the one you expect to have the highest intensity in all your channels).
12. Under illumination Click "Set intensity" to adjust BF intensity.
13. Check for saturation in all channels in the histograms. A peak at 4095 is a sign of saturation. If you have a peak at 4049, create a gate around this peak and turn down laser power until a maximum of 1% of events are saturated. If you look for rare events, this percentage should

be even lower. You can perform pre-gating and only look at your cells of interest in this plot, to ensure you do not turn down laser power too much.

14. You may manually adjust display settings for each channel by clicking on a channel. This is, however, just a visual effect and will not necessarily reflect your final results;

For each channel needed in your experiment press “display settings” -> “set range to pixel data” and adjust the bar to exclude background. When finished, click “OK”.

Be aware that this adjustment does not always result in optimal images because the cells are in a flow.

15. Create your gating strategy

Suggestions for collection gates:

- i. Gate on focused cells in a Gradient RMS Ch01 histogram
  - ii. Create an Area M01 vs Intensity Ch06/Ch12 (SSC) plot to gate on a subpopulation of cells (maybe you need to change the SSC scale to linear).
  - iii. Create an Area M01 vs Aspect Ratio M01 plot to gate on singlets.
  - iv. Create an Intensity Ch“A” vs Intensity Ch“B” plot to gate on a subpopulation of cells.
16. Enter the number of cells you want to acquire in the box after Count and select the population from the dropdown. To count one population but limit the collection to another population, click on the bracket to break the link and enable the Collect
  17. Press “Acquire”.
  18. After collecting your data, press **Return** (NOT load), in the upper right corner to return the rest of your sample back into your tube.
  19. ***If you have similar samples (same cell type and same fluorochromes) which should be run another day – save a template (this will save laser settings, speed, magnification, plots and gates. (file -> save template -> Desktop -> New export folder – “your name”)***
  20. To avoid carry-over from one sample to another or to remove DNA;
    - load 200 µl Na-hypochlorite and press load (not return).
    - load 200 µl water and press load (not return).

### **Compensation**

If you use more than one fluorochrome you need to compensate your data. Laser power (except 785 nm SSC laser) should be the exact same for your compensation controls as they are for your samples.

You can either use the compensation wizard or do it manually. A compensation matrix can be applied directly in INSPIRE or afterwards in IDEAS software.

*Before running the compensation samples, clean the instrument as described above if you have DNA stain in your panel*

## 2. **Manual compensation**

- activate ALL channels, deselect brightfield and turn off the SSC laser.
- Run all your single stained samples.
- Remember to turn on SSC and brightfield and turn off the channels you do not need after running your single stained samples.

## 3. **Compensation wizard;**

- Go to “Compensation” and choose “create matrix”.
- A new window opens. Press load and load a single stained sample
  - i. Order of running your samples:
    1. Single colour fluorescence controls (no DNA dye)
    2. Single colour DNA dye control
- Press “next” when possible. In the next window, choose the right channel for your fluorochrome and click “next”.
- Type the name of your sample (ex. Ch2\_CD45FITC). Draw a gate “R1” containing all positive events in an “uncompensated Intensity\_MC\_ChX” plot, and choose to collect 500 “R1” events.
- Press “Acquire”
- Press “return” to return your sample to the eppendorf tube.
- When all single stained samples have been acquired press “exit” in the lower left corner of the compensation window (you can either save the compensation matrix or you can create a compensation matrix in IDEAS later using the compensation files just collected). The compensation matrix window will close.

## **Samples**

4. If not already done, run your remaining experimental samples.
5. To avoid carry-over from one sample to another and to remove DNA dye;
  - load 200 µl Na-hypochlorite and press load (not return).

- load 200 µl water and press load (not return).

When finished, transfer your data to your AU-drive and delete your .rif files on this computer afterwards.

6. Check iLab – are you the last user of the day?
7. If you are the last user of the day– follow the “shutdown procedure”.
8. If you are **not** the final user of the day – follow the “user change procedure”.

### Trouble shooting

If your *event rate is decreasing* it may be caused by

- 1) cell sedimentation (slowly decreasing event rate). If this is the case you have three options:
  - Filter your sample through a 50-70µm mesh strainer.
  - Return your sample (this will dilute your sample with 15µl sheath). Then load your sample again and acquire.
  - Pause acquisition -> prime -> resume acquisition (you will loose approximately 10µl sample using this procedure).
- 2) a blockage
  - choose “Purge sample load line”.
- 3) a too concentrated sample
  - dilute your sample.

If you have *aggregates* in your sample you should filter the samples before loading it to the instrument (50-70µm mesh strainer). Aggregates can cause bubbles, which will affect both focus and signal.

If *bubbles* are detected or you refilled any of the fluidics

- a. go to “Instrument” -> “purge bubbles”
- b. go to “Instrument” -> “purge sample load line”
- c. go to “Instrument” -> “prime”

Some samples have an abundance of small particulate *debris*. These can be eliminated by gating or by using Squelch to reduce the sensitivity of object detection. As opposed to gating debris away from cells, squelching debris can prevent INSPIRE crashes related to overburdening the computer processor with an abnormally high event rate. Squelch should only be used if the rate of total objects per second reaches 4000. Squelch values range from 0 to 100; increasing the value decreases object detection sensitivity.

1. Choose “All” in the image gallery.
2. Observe the relative proportion of cell to debris images appearing in the imaging area and the event rate (Total/Sec under Acquisition Status).
3. On the Advanced Setup -Acquisition tab, increase the Squelch value until the observed proportion of cells to debris increases in the imaging area.
4. Observe the Total/Sec event rate on the Setup tab under Acquisition Status. If it is still greater than 500, repeat step 2.